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Epidermal growth factor (EGF) family of ligands bind to a number of related receptors and stimulate mitogenesis in mammary cells. Signal transduction downstream of the EGF family of receptors involves activation of the GTPase Ras and its effectors. *Rsu-1*, a cDNA identified as an inhibitor of Ras-induced transformation, was HA-epitope tagged and introduced into expression vectors. The HA-*Rsu-1* vectors were transfected into MCF7 breast carcinoma cell lines. Transfectants were selected and tested for response to EGF and serum stimulation. HA-Rsu-1 transfectants had similar anchorage dependent growth rates as the control cell lines, but exhibited a reduction in anchorage independent growth. Analysis of Ras-dependent kinase pathways in the *Rsu*-1 transfectants following stimulation of starved cells with EGF or serum revealed that expression of HA-Rsu-1 resulted in an increase in activation of Erk-2 kinase and a decrease in activation of p54 Jun kinase. In addition, Rho-dependent Rho alpha kinase was inhibited but Rac and Cdc42-dependent αPak serine threonine kinase was not affected by Rsu-1 expression. Activation of AKT kinase by serum and EGF was unaffected by Rsu-1 expression. These results focus attention on the role of Rsu-1 inhibited pathways in Ras signaling in MCF7 breast carcinoma cell line.

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INTRODUCTION

Epidermal growth factor (EGF) family of ligands bind to a number of related receptors and stimulate mitogenesis in mammary cells. Signal transduction downstream of the EGF family of receptors involves activation of the GTPase Ras and its effectors. Expression cloning to isolate inhibitors of Ras-induced transformation identified the Rsu-1 cDNA [4]. Previous experiments demonstrated that the expression of the Rsu-1 gene in NIH3T3 fibroblasts under the control of a heterologous promoter could suppress transformation by v-Ras but not v-Raf, v-Mos, or v-Src oncogenes [4], and experiments in our laboratory have determined that the Rsu-1 suppressor inhibits EGF- and TGFa-induced anchorage independent growth of NOG8 mouse mammary cells. *rsu-1* is a highly conserved single copy gene which encodes a protein of 33kD. p33 Rsu-1 contains a series of 23 amino acid leucine-based amphipathic repeats homologous to the repeats found in yeast adenylyl cyclase in the region through which Ras activates adenylyl cyclase in *Sa. cerevisiae* [4]. This suggested that Rsu-1 might interact with Ras or Ras-regulatory or -effector molecules. Transfection of a Rsu-1 vector into NIH3T3 cells and PC12 cells resulted in an increase in signal to Raf-1 and Erk-2 kinase and a decrease in signal transduction to Jun kinase [8]. These results demonstrate that Rsu-1 can alter signal transduction downstream of Ras.

Signal transduction "downstream" of Ras depends on the association of Ras with its effector proteins. Several proteins have been identified which associate with Ras in a GTP-dependent manner. These include Raf-1, RasGAP, p110 subunit of PI-3-kinase, Rin-1, Mek kinase 1, protein kinase C zeta, and RalGDS; in the case of several of these proteins (i.e. Raf-1, RasGAP, p110 PI3kinase) signal transduction pathways activated as a result of interaction with Ras have been characterized. Activation of Ras effectors can lead to phosphorylation and activation of kinases, cytoskeletal proteins and transcription factors. For example, activation of Raf-1 results in specific phosphorylation of Mek and Erk-2 [14], and use of dominant negative Erk demonstrated that inhibition of this pathway interefered with Ras transformation in fibroblasts [3]. The small G proteins Rac and Rho are activated independently of Raf-1 by an as yet uncharacterized mechanism in response to activated Ras, and very recent evidence suggests that they play a crucial role in growth and tumorigenesis of epithelial cells. Studies using dominant negative Rho and Rac indicated that inhibition of Rho and/or Rac pathways prevented transformation by Ras, suggesting that these proteins regulate pathways essential for Ras transformation [6, 12, 13]. Activated Rac and cdc42 induced transformation in epithelial cells [7], whereas activation of the Raf-1/Erk pathway alone was not sufficient to transform rat intestinal and MCF10A epithelial cell lines [10]. These results, along with studies describing properties of transformants produced by specific effector mutants of Ras [1, 7, 15], have led to the conclusion that several pathways "downstream" of Ras contribute to transformation of cells in response to activated Ras. Most interestingly, activation of Rho and Rac appears to be responsible for induction of anchorage independent growth and tumorigenicity in epithelial cells [7]. Because cdc42Hs and Rac have been shown to be required for the activation of the stress-activated or Jun kinase [2, 9, 11], and RhoA has been shown to be necessary for activation of the SRF by serum and other agents that act through the pertussis-sensitive G protein pathway [5], it appears that the small G proteins may exert their effects via transcriptional activation.

Because pathways dependent on Rac and Rho are important for maintaining the transformed state, inhibitors of this pathway should prove especially useful in further dissection of growth control pathways and in developing antitumor therapies. Because the Rsu-1 suppressor has been shown to inhibit transformation and events dependent on Rac and Rho [8] we are investigating its role in the growth regulation of breast carcinoma.

Current experiments are designed to assess the ability of Rsu-1 to regulate pathways "downstream" of Ras which are activated by members of the EGF receptor family in human mammary carcinoma cells. Rsu-1 transfectants have been prepared in the MCF 7 cell line for analysis of response to EGF and estrogen. The Rsu-1 transfectants have been compared to vector control transfectants for anchorage-dependent and -independent growth. The effect of Rsu-1 on the Ras-induced activation of downstream effectors (RasGAP, Raf-1, Mek kinase, and PI3-kinase) has been determined by analyzing the kinases regulated by specific Ras effectors following stimulation of the cells. The kinases include: MAP kinases Erk-2, Jun kinase, p38 kinase, and Akt kinase (protein kinase B), αPak, and Rho kinase. The effect of Rsu-1 expression on activation of myc transcription has been examined. The influence of estrogen on the regulation of the EGF-dependent pathways has been tested in analogous experiments, however the results are not yet complete. Finally, transfectants in the MCF10A and MDA-MB468 cells are being constructed. The difficulties using vectors for the constitutive expression of Rsu-1 in MCF10A cells will be described along with a description of the inducible vector system now being employed.

BODY:

Several vectors have been used for the expression of p33Rsu-1 in human breast epithelial cell lines. These vectors include: p3v36 which contains the *RSU-1* cDNA under the control of a MT-1 promoter in a retroviral vector [Cutler, 1992 #1], p3v65 which contains an HA-tagged version of the *RSU-1* cDNA under the control of a RSV promoter in a vector containing an SV40 origin of replication [Masuelli, 1996 #371], and p3v64 which contains an HA-tagged *RSU-1* cDNA in a retroviral vector under the control of a MT-1 promoter. MCF 7 and MCF10A cell lines have been transfected or infected with the above vectors and the appropriate "empty" control vectors. Following selection in G418 single colonies have been isolated using cloning cylinders, expanded into cell lines and screened for *RSU-1* RNA expression. In the case of transfectants containing the p3v36 or p3v64 vectors the transfectants were assayed in the presence and absence of Cd++ which results in induction of transcription from the MT-1 promoter. The following table lists transfectants prepared to date.

MCF 7	p1521	control vector	transfectants obtained
MCF7	p3v36	*Rsu-1* vector	transfectants obtained
MCF7	p521	control vector	transfectants obtained
MCF7	p3v65	*Rsu-1* vector	transfectants obtained

MCF10A	p1521	control vector	transfectants obtained
MCF10A	p3v36	*Rsu-1* vector	transfectants obtained*
MCF10A	p3v64	*Rsu-1* vector	transfectants obtained*
MCF10A	p521	control vector	transfectants obtained
MCF10A	p3v65	*Rsu-1* vector	transfectants not obtained

* No clones expressed Rsu-1 in response to Cd++.

It is interesting to note that the transfection of MCF10A cells with vector encoding *RSU*-1 cDNA under the control of a constitutive promoter did not result in the isolation of any G418 resistant colonies. It appears that the growth suppressive properties of Rsu-1 are sufficient to inhibit the growth of normal cells expressing this protein at high level. MCF10A clones derived from infection of cells with a retroviral vector containing Rsu-1 under the control of an inducible MT-1 promoter have been isolated, but no clones express vector-derived RNA in response to Cd++ . Hence, another inducible system, the ecdysone responsive promotor vector, is being used (see below).

MCF 7 TRANSFECTANTS

Transfectants in the MCF 7 cell line were analyzed for expression of *Rsu-1*, the effect of *Rsu-1* expression on biological properties of the cells and signal transduction in the Ras pathway. The results shown below are those obtained from MCF 7 transfectants derived following transfection of p3v65 which encodes HA-tagged Rsu-1.

Expression of *RSU-1* RNA and protein in MCF 7 cell clones . The last report included data demonstrating that the 3V65 MCF7 clones expressed vector-derived Rsu-1 specific RNA and protein. Vector-derived Rsu-1 RNA was detected by Northern blotting. Cell lysates were analyzed by Western blotting for the expression of p33 HA-Rsu-1 using a mouse monoclonal antibody directed against the HA epitope tag (clone 12CA5). The results indicated that the HA-Rsu-1 was detected in all but one transfectant. The levels of HA-Rsu-1 were somewhat higher in two of the clones, 3v65-9B and 3v65-14B. The levels of Ras, Rac, Rho and RasGAP are similar in all clones and are similar to the levels in the control and parental cell lines.

Biological Properties of MCF7 clones expressing HA-Rsu-1 (figure 1 and 2). The growth properties of the Rsu-1 transfectants were compared to those of the control cell lines. Anchorage dependent growth of MCF7 cells expressing HA-Rsu-1 was compared to wild type MCF7 cells and a vector control cell line. The results, in the form of a growth curve (figure 1), demonstrate that while the plating efficiency of the clones was slightly lower than the control cell lines, the growth rates were similar. Anchorage independent growth was also tested and the results indicated that the clones exhibit a significant reduction in anchorage independent growth compared to the vector control transfectant and untransfected MCF 7 cell lines (figure 2).

The effect of Rsu-1 expression on the activation of the Ras signal transduction pathway (figures 3, 4, and 5). Treatment of cells with growth factor, serum or TPA was used to activate Ras. Then the

activation of specific kinases which are dependent on specific Ras effectors was tested in control as well as HA-Rsu-1 expressing clones.

The activation of Erk2 was tested in serum starved HA-Rsu-1 clones and control cell lines. Cells were stimulated for 7.5 minutes with Epidermal Growth Factor (EGF) (100ng/ml) or for 10 minutes with TPA (100ng/ml). The cells were lysed and Erk2 was immunoprecipitated and assayed in an *in vitro* kinase assay using myelin basic protein (MBP) as a substrate [Masuelli, 1996 #371]. MBP was separated by SDS-PAGE, transferred to filter, and the labeled MBP on the filter was quantitated (figure 3). In agreement with results obtained by us in other cell types there is an increase in the activation of Erk2 in response to EGF in all clones, and an increase in response to TPA in the clones which express the highest levels of HA-Rsu-1.

The results of the activation of Jun kinase by growth factor (EGF) has been tested in an *in vitro* kinase assay. Jun kinase was immunoprecipitated from lysates of serum starved cells following EGF stimulation. The kinase activity was determined using a GST-cJun fusion protein as a substate. The results (figures 4 and 5) indicated that in serum starved MCF 7 cells there was constitutive Jun kinase activity which was modestly enhanced by the addition of EGF. However, the Rsu-1 clones exhibited a lower amount of Jun kinase activity which was stimulated by EGF in only one of the clones tested. Our data included in the last report suggested that the Jun kinase activity in these cells was a result of expression and activation of p54 Jun kinase and not p46 Jun kinase. These experimental results demonstrating an inhibition of Jun kinase activity are in agreement with those reported by us previously.

The activity of Rho alpha kinase following EGF stimulation of MCF7 cells and the transfectants was tested. Rho kinase is a 180 kD serine threonine kinase which associates with the GTP bound form of the Rho A GTPase and its activation correlates with the transforming functions of Rho . Rho kinase activity was determined in an immune kinase assay using Histone H1 as a substrate. Our results (figure 4 and 5) indicated that Rho kinase activity could be stimulated by EGF in MCF7 cells but not in the Rsu-1 transfectants. Pak kinase (p21 activated kinase) complexes with Rac and Cdc42 in the GTP-bound state. The activation of Pak activity was detected in an immune kinase assay using an antibody which reacts with α, β,γ Pak and using Histone H1 as a substrate. The results, also shown in figure 4, indicate that the level of Pac kinase activity was unaffected by EGF in both the control and the transfectants.

The activation of the Akt kinase was tested in response to both serum (results included in the last report) and EGF (data not shown). The AKT kinase activation is dependent on the Ras effector phosphatidyl inositol-3 kinase. AKT kinase activity can be activated in serum starved cells by the addition of serum to the cultures. The activation of AKT was measured 30 minutes after the addition of serum by Western blotting of total cell protein with antibodies which react with the phosphorylated version of AKT kinase (New England Nuclear). The results demonstrated that the HA-Rsu-1 expressing clones had a slightly lower level of phosphorylated AKT kinase following serum starvation. However, phosphorylation of AKT kinase was achieved in the clones in response to both serum and EGF. These results suggest that the activation of phosphoinositol-3-

kinase by Ras-dependent and -independent mechanisms, and the subsequent activation of AKT kinase was achieved. Rsu-1 expression did not disrupt this pathway.

Regulation of Myc transcription in Rsu-1 transfectants (figure 6). Our previous unpublished studies had indicated that the expression of Rsu-1 inhibited the level of myc transcription in response to serum and growth factor. The induction of myc transcription following serum stimulation for 0, 1, and 4 hours in MCF7 and Rsu-1 MCF7 transfectants was determined by Northern blotting (figure 6) and RT-PCR (data not shown). Induction of myc RNA by serum was elevated to approximately 60% the level in the Rsu-1 transfectants compared to the control cells. This result supports our previous findings in other cell backgrounds. Recent studies demonstrated the stabilization of myc protein by Ras expression [17] and the regulation of myc RNA stability by a RasGAP binding protein [16]. These studies suggest that additional examination of the mechanism by which myc RNA levels are reduced in Rsu-1 transfectants would be useful.

Activation of Ras-dependent pathways in the absence of estrogen. The effect of estrogen on the stimulation of Ras-dependent pathways in the MCF7 and Rsu-1 MCF7 transfectants is underway. Because the analysis of the data for those experiments is not complete, no data will be reported at this time. However, the experiments performed include the comparison of activation of Ras-dependent kinase pathways in the presence and absence of estrogen. In addition, the activation of the early response gene transcription is being compared in the presence and absence of estrogen. Finally, the regulation of growth and growth arrest by cdk inhibitors in the presence and absence of estrogen is being determined.

MCF10A TRANSFECTANTS USING ECDYSONE-INDUCIBLE PROMOTOR VECTORS.

As described above the transfection of MCF10A cells has not yielded transfectants which express HA-Rsu-1 constitutively or in response to Cd++ induced transcription from the MT-1 promoter. Clones of MCF10A have been obtained readily with the control (empty) vectors, and clones which contain vectors carrying the HA-Rsu-1 cDNA have been isolated by both transfection and retroviral infection. The latter clones, >20 in number, do not activate transcription of HA-Rsu-1 in response to Cd++. Because we have had success with the MT-1 promotor vectors in MCF10A cells for the expression of TGFalpha and c-ErbB2, it appears that the current problems are related to the expression of a growth inhibitory cDNA. Therefore, we have initiated use of the ecdysone-inducible promotor vector in these cells. Ecdysone is an insect steroid hormone which recognizes a receptor not found in any mammalian cells [18]. The first step in the use of this system was the construction of cell lines expressing the two subunits of the ecdysone receptor, one of which has been engineered to contain the DNA binding domain of VP16. The sequences encoding the two subunits of the receptor are contained in the vector, pVgRXR (commercially obtained from In Vitrogen, Carlsbad CA). Together these subunits serve to activate transcription in the promotor of the expression vector, pIND, in response to muristerone A/ ponasterone. The HA-Rsu-1 cDNA has been introduced into the pIND vector. The resulting plasmid, p3V77, has been functionally tested in NIH3T3EcR cells which contain the pVgRXR vector. Clones of MCF10A expressing the receptor

have been isolated and have been transfected with plasmids containing the ecdysone-responsive promotors. Screening for inducible clones expressing HA-Rsu-1 is underway.

CONCLUSIONS:

Rsu-1 transfectants have been successfully prepared in two cell lines, MCF7 and MCF10A. The expression of Rsu-1 does not have a potent effect on the anchorage dependent growth rate in MCF7 breast carcinoma cell line, but Rsu-1 does inhibit anchorage independent growth. Analysis of the influence of Rsu-1 expression on Ras-dependent pathways has indicated that there is an increase in the activation of Erk 2 in response to EGF and TPA. There is a decrease in the activation of Jun kinase in response to serum and EGF, but little change is detected in the PI-3-kinase dependent activation of AKT kinase in respnse to serum and EGF. However, activation of Rho alpha kinase by EGF is inhibited in the transfectants, but there is little effect on the activity of the Rac and Cdc42 dependent αPak serine threonine kinase by Rsu-1 expression. The results indicate that the expression of Rsu-1 does influence signal transduction "downstream" of Ras. In particular, there is inhibition of Rho dependent signaling events. These transfectants will allow the continued delineation of the role of various Ras effectors in the activation of downstream kinases in human mammary carcinoma cell lines. Correlation of biochemical changes to inhibition of specific biological properties should point to the specific signal transduction pathway(s) responsible. At present the role of Rsu-1 in altering signal transduction in the Ras pathway in response to growth factor and serum has been tested. The influence of estrogen on alteration of Ras pathway by Rsu-1 will also be determined. Further experimentation will allow determination of the effect of the number of EGF receptors, and hence the level of activation of the Ras pathway, on the influence of *Rsu-1* on downstream kinase pathways.

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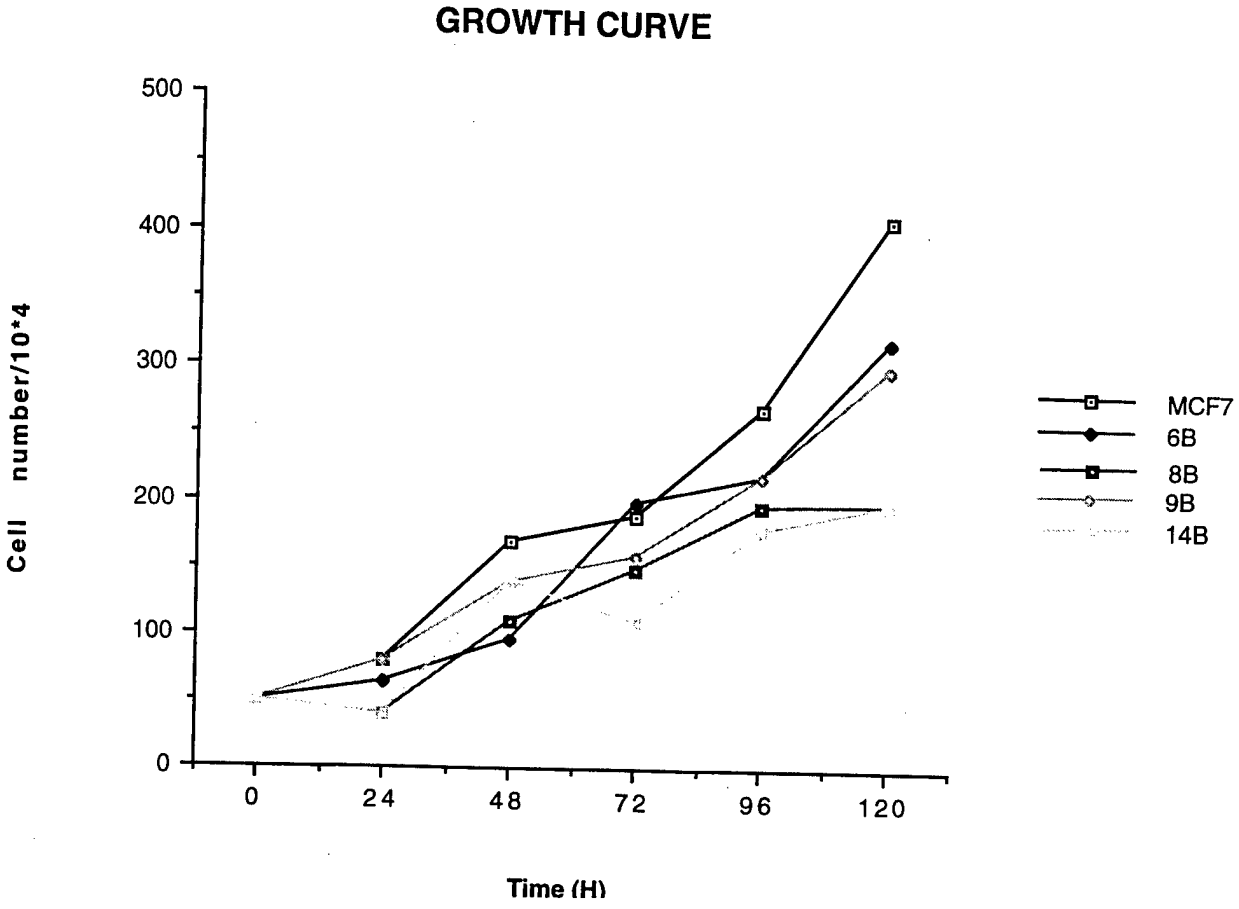


Figure 1

Anchorage dependent growth of MCF-7 cells and HA-Rsu-1 transfectants were compared. The results are shown in the curve. MCF-7 and Rsu-1 transfectants were plated at 50,000 cells per 60 mm tissue culture dish in EMEM with 10% FBS. The cells were counted after 24,48, 72, 96 and 120 hours. The number of cell reported at each time point was the average of two plates.
All data shown are representative of three independent experiments.

Figure 2

Biological Properties of MCF7 clones expressing HA-Rsu-1

MCF7 cell lines have been transfected with the vector p3v65, which contains an HA-tagged version of the RSU-1 cDNA under the control of a RSV. MCF7 have been transfected also with appropriate "empty" control vectors. Following selection in G418 single colonies have been isolated using cloning cylinders, expanded into cell lines. Cell lysates were analyzed by Western Blotting for the expression of p33 HA-Rsu-1 using a mouse monoclonal antibody directed against the HA epitope tag. Clones 3v65-6B, 8B, 9B and 14B which express HA-tagged Rsu-1 were tested for anchorage independent growth with MCF7 contol cells. All clones produced fewer colonies than the vector control transfectants and untransfected MCF7 cell lines. The results of four separate determination were averaged and the percentage of MCF 7 cells giving rise to colonies was normalized to 100%. The values for the 3v65 cell lines are expressed as the percentage of the MCF7 value.

CELL LINE	
MCF7	100%
3v65-6B	44.5%
3v65-8B	33.2%
3v65-9B	11%
3v65-14B	27.2%

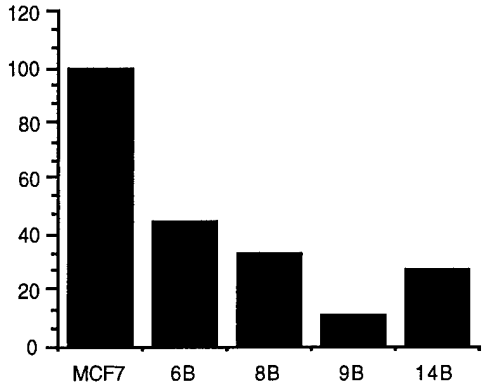
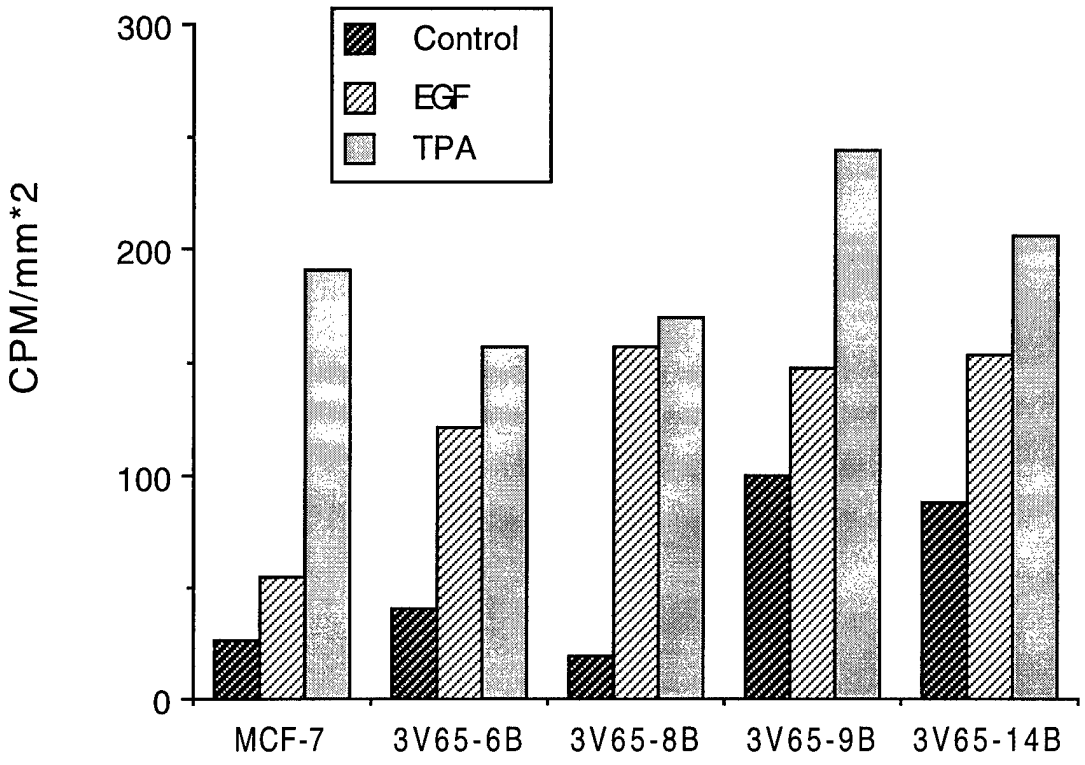


Figure 3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200
100
0
CPM/mm*2
Control
EGF
TPA
MCF-7
3V65-6B
3V65-8B
3V65-9B
3V65-14B

Figure 4. Stimulation of Rho kinase by EGF

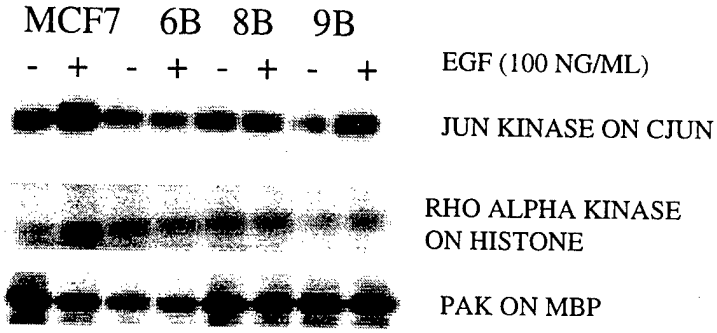

Figure 5

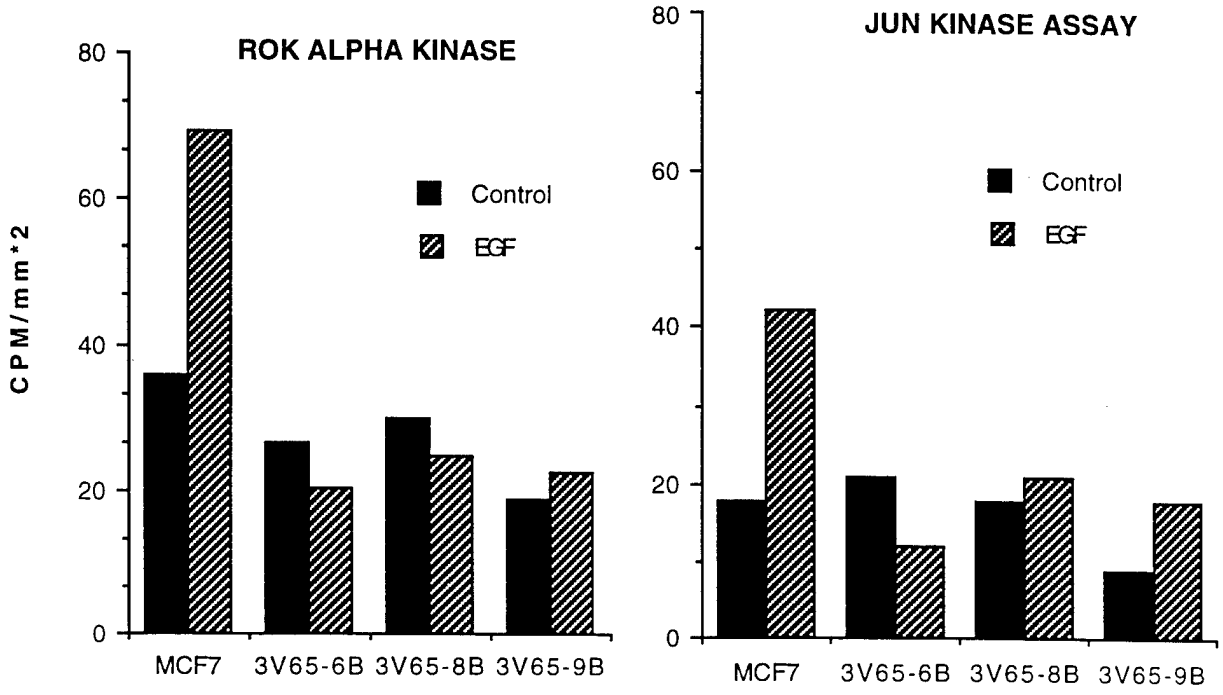

FIGURE 6 . c-myc expression in 3v65 Rsu-1 transfectants

The expression of c-myc RNA in response to serum stimulation was analyzed in MCF7-3v65-Rsu-1 transfectants. Cells were serum starved for 16 hours then stimulated with 20% serum for 0, 1, 4 hours and RNA was isolated and used for Northern blotting. The amount of c-myc RNA at each time point was quantitated. GPDH was used as a control RNA signal. As shown below the activation of c-myc transcription in response to serum in the transfectants was approximately 60% of the MCF7 control cell line.